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Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach

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Summary

The biodiversity of arbuscular mycorrhizal fungi (AMF) communities present in five Sardinian soils (Italy) subjected to different land-use (tilled vineyard, covered vineyard, pasture, managed meadow and cork-oak formation) was analysed using a pyrosequencing-based approach for the first time. Two regions of the 18S ribosomal RNA gene were considered as molecular target. The pyrosequencing produced a total of 10924 sequences: 6799 from the first and 4125 from the second target region. Among these sequences, 3189 and 1003 were selected to generate operational taxonomic units (OTUs) and to evaluate the AMF community richness and similarity: 117 (37 of which were singletons) and 28 (nine of which were singletons) unique AMF OTUs were detected respectively. Within the *Glomeromycota* OTUs, those belonging to the *Glomerales* order were dominant in all the soils. *Diversisporales* OTUs were always detected, even though less frequently, while *Archaeosporales* and *Paraglomerales* OTUs were exclusive of the pasture soil. Eleven OTUs were shared by all the soils, but each of the five AMF communities showed particular features, suggesting a meaningful dissimilarity among the *Glomeromycota* populations. The environments with low inputs (pasture and covered vineyard) showed a higher AMF biodiversity than those subjected to human input (managed meadow and tilled vineyard). A reduction in AMF was found in the cork-oak formation because other mycorrhizal fungal species, more likely associated to trees and shrubs, were detected. These findings reinforce the view that AMF biodiversity is influenced by both human input and ecological traits, illustrating a gradient of AMF communities which mirror the land-use gradient. The high number of sequences obtained by the pyrosequencing strategy has provided detailed information on the soil AMF assemblages, thus offering a source of light to shine on this crucial soil microbial group.

Introduction

Metagenomic studies have recently provided new approaches that shed light on microbial communities in a variety of environments – e.g. sewage digestors, dental plaque, termite gut, deep mine drainages – (Schloss and Handelsman, 2005; Hugenholtz and Tyson, 2008). Metagenomics provides a relatively unbiased view not only of the community structures (species richness and distribution), but also of the functional potential of a community (van Elsas *et al.*, 2008).

In this context, DNA pyrosequencing with the 454 GS-FLX platform, a rapid and relatively inexpensive sequencing technology that produces hundreds of thousands of short sequences, is being used more and more for prokaryotic metagenomic studies (Edwards *et al.*, 2006; Warnecke *et al.*, 2007). This technique has recently been used to enumerate and compare soil bacterial diversity (Roesch *et al.*, 2007; Fulthorpe *et al.*, 2008). However, so far, no pyrosequencing-based studies have been focused on soil fungi. Fungi are a crucial component of soil microbial communities, in which they function as decomposers, pathogens and mycorrhizal mutualists. Among the members of this last group, arbuscular mycorrhizal fungi (AMF) are the most important symbionts in many ecosystems (Oehl *et al.*, 2003; Öpik *et al.*, 2008; Toljander *et al.*, 2008). Arbuscular mycorrhizal fungi have been separated from all other major fungal groups and grouped in a monophyletic clade, named *Glomeromycota* (Schüßler *et al.*, 2001), within which four orders (*Glomerales*, *Diversisporales*, *Archaeosporales* and *Paraglomerales*) have been described (Krüger *et al.*, 2009).

Arbuscular mycorrhizal fungi play a key role in supplying phosphorus to plants, which in return receive plant carbon assimilates (Girlanda *et al.*, 2007; Smith and Read, 2008). In addition to an improvement in plant nutrition, AM fungi protect their hosts from pathogens (Pozo and Azcon-Aguilar, 2007) and affect plant growth traits (Artursson *et al.*, 2006). Furthermore, it has been suggested that mycorrhizal fungal diversity is a determinant of plant diversity (van der Heijden *et al.*, 1998; van der Heijden and Scheublin, 2007). This concept has been a driving force in the investigation of AMF community diversity in different environments (Öpik *et al.*, 2006; Vallino *et al.*, 2006) and up to 34 different AMF taxa have been found in a single habitat (Öpik *et al.*, 2008). Arbuscular mycorrhizal fungi taxa have a specific multidimensional niche that is determined by the plant species that are present at a site and by edaphic factors such as pH, moisture content, phosphorus (P) and nitrogen (N) availability. As a result, large between- and within-site variations in the composition of AMF communities have been described (Burrows and Pfleger, 2002; Klironomos and Hart, 2002). However, a basic weakness of AMF community analyses is that many studies have been exclusively focused on roots, without considering that the extra-radical phase is at least as important as the intraradical mycelia for the nutrient supply of the host plant (Horton and Bruns, 2001). The current interest in extra-radical mycelium is increasing (Croll *et al.*, 2009) because the occurrence of anastomoses between genetically distinct mycelia suggests the existence of mycelial networks which connect different plant–host roots in soil (Young, 2009).

Here we present, to our knowledge, the first and the largest AMF soil-based sequence data set, obtained from high-throughput DNA pyrosequencing. Our investigation had two objectives: first to test the potentiality of pyrosequencing to investigate the diversity of AMF in a Mediterranean ecosystem, and second to characterize and compare AMF communities along a land-use gradient. The study area, located in Berchidda (Sardinia, Italy), in the past was covered by cork-oak forests (*Quercus suber* L). Over the years this vegetation has been subjected to intense usage for the extraction of cork and pasture. Therefore, today, it is possible to find different land-use units close together: tilled vineyard (coded TV), non-tilled cover cropped vineyard (CV), managed meadow (MM), pasture or grassland (PA) (dominated by pasture or grass species with a low tree density) and cork-oak formation (CO) (dominated by shrub cover and distributed cork-oak trees).

Using two couples of primers we obtained well over 10 000 eukaryotic rRNA 18S gene fragments from five Sardinian soils. The sequences were classified into operational taxonomic units (OTUs). The OTUs were created using the DOTUR software, which grouped the 18S rDNA sequences according to different similarity levels. This study has been focused on OTUs defined at the ≥97% similarity level ($OTU_{0.03}$) to characterize the AMF communities. Although this distance cut-off is arbitrary and could be considered controversial, it has been used in many studies (Schloss and Handelsman, 2005; Huber *et al.*, 2007), and it facilitates comparisons with similar studies based on cloning and sequencing (O'Brien *et al.*, 2005). The sequence data sets allowed us to make comparative analyses of AM fungal communities, taxa richness and coverage estimates as well as to obtain an overall description of the order-level diversity.

Results

Overall taxonomic richness

A total of 10 924 sequences were obtained: 6799 and 4125 with the AMV4.5NF/AMDGR and NS31/AMmix primer pair respectively. Only fragments of ≥ 230 bp in length were analysed for AMV4.5NF/AMDGR and of ≥ 250 bp for NS31/AMmix, leading to a total of 4192 sequences: 3189 (average length: 258 bp) and 1003 (average length: 274 bp) for AMV4.5NF/AMDGR and NS31-AMmix respectively (Fig. 1A).

A

Organisms	AMV4.5NF/AMDGR		NS31/AMmix	
	Sequence Numbers	Rate (%)	Sequence Numbers	Rate (%)
Glomeromycota	2438	76.45	377	37.59
Ascomycota	4	0.13	448	44.67
Basidiomycota	355	11.13	35	3.49
Zygomycota	7	0.22	13	1.30
Chytridiomycota	103	3.23	1	0.10
Non Identified Fungi	109	3.42	73	7.28
Other Eukaryotes	79	2.48	1	0.10
Unknown Organism	94	2.95	55	5.48
	3189	100	1003	100

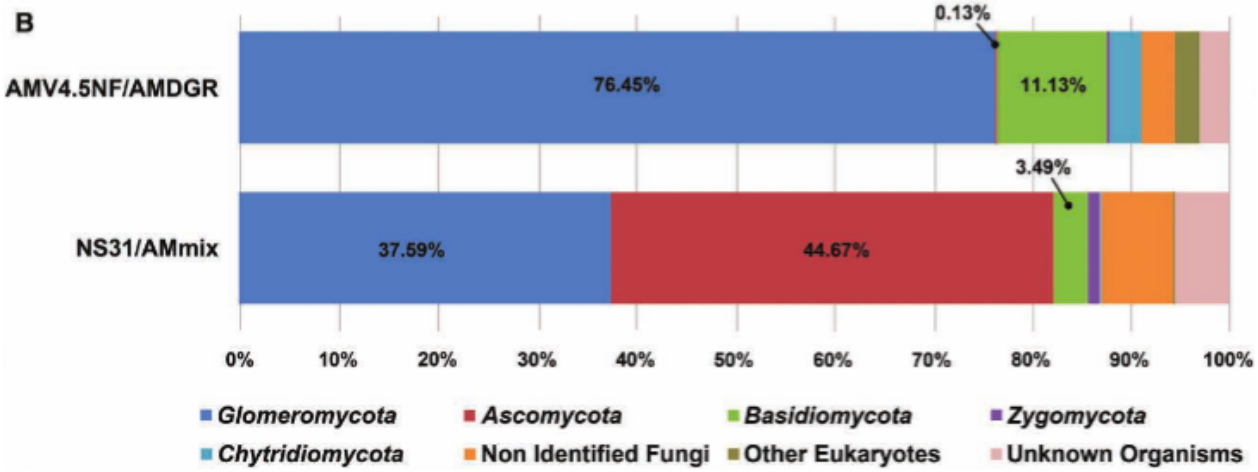

Figure 1. Table (A) reports the number of sequences of each fungal phylum detected with the AMV4.5NF/AMDGR and NS31/AMmix primer pairs in all the Sardinian soils sampled. Picture (B) shows the overall and proportional distribution of the fungal phyla detected. Glomeromycota *(blue) and* Ascomycota *(red) were the most abundant taxa found with the two couples of primers respectively.*

In spite of the supposed AM primer specificity, some 'contaminant' sequences were detected, belonging to taxa different from *Glomeromycota*. The taxonomic distribution of the 18S sequences obtained with each primer pair in the Sardinian soils is shown in Fig. 1A and B. The majority of eukaryotic sequences (2438 corresponding to 76.45% of the total) amplified with AMV4.5NF/AMDGR belong to *Glomeromycota*. With the same primer pair, the *Basidiomycota* relative abundance was 11.13%, while only 0.13% of the sequences was assigned to *Ascomycota*. Among the 1003 NS31/AMmix sequences, 448 sequences, corresponding to 44.67% of the total sequences, instead referred to *Ascomycota* and only 37.59% of the sequences (377) to *Glomeromycota*. The *Basidiomycota* relative frequency was 3.49%. The other two fungal phyla (*Chytridiomycota* and *Zygomycota*) were recovered less by both primer pairs: 0.10% with NS31/AMmix and 3.23% with AMV4.5NF/AMDGR, and 0.22% (AMV4.5NF/AMDGR) and 1.30% (NS31/AMmix) respectively (Fig. 1).

Arbuscular mycorrhizal fungi community richness

Overall, 2815 *Glomeromycota* sequences were obtained: 2438 and 377 with AMV4.5NF/AMDGR and NS31/AMmix respectively. Considering each soil type, AMF sequences ranged from 188 (CO) to 1275 (PA) for the AMV4.5NF/AMDGR primers and from 12 (CO) to 200 (PA) for the NS31/AMmix primers (Table 1). Despite the different sequence numbers obtained, there was good agreement between the amplification results obtained from the two couples of primers, possibly reflecting an actual higher presence of *Glomeromycota* in the PA soil and a substantially lower occurrence in the CO soil.

Table 1. Number of *Glomeromycota* sequences detected with the AMV4.5NF/AMDGR and NS31/AMmix primer pairs.

	Number of *Glomeromycota* sequences	
	AMV4.5NF/AMDGR	**NS31/AMmix**
Tilled vineyard	429	17
Covered vineyard	292	66
Managed meadow	254	82
Pasture	1275	200
Cork-oak formation	188	12
Total	2438	377

1. Numbers refer to the ≥ 230 bp sequences for the first couple of primers and to ≥ 250 bp for the second one.

The obtained AMF sequences were grouped into OTUs, representing groups of sequences at different similarity levels. Rarefaction curves were used to depict the effect of percentage similarity on the number of identified OTUs (Fig. 2). The patterns of the rarefaction curves relative to the two targeted 18S rDNA regions, amplified by the two primer sets, were similar: at high similarity levels (97%, $OTUs_{0.03}$ and 95%, $OTUs_{0.05}$), neither curve reached the plateau, while at low similarity levels (85%, $OTUs_{0.15}$ and 80%, $OTUs_{0.20}$) both the curves reached the plateau. This result is confirmed by a comparison of the observed (rarefaction index) and estimated (non-parametric ACE-Abundance base Coverage Estimator and Chao1 indices) OTUs at different levels of similarity: at the 80% and 85% sequence similarity levels, the number of observed OTUs was found to be equivalent, or close to those of the number of estimated OTUs (ACE and Chao1 indices), while at higher similarity levels, the ACE and Chao1 richness values were relatively far from the observed ones (Table 2).

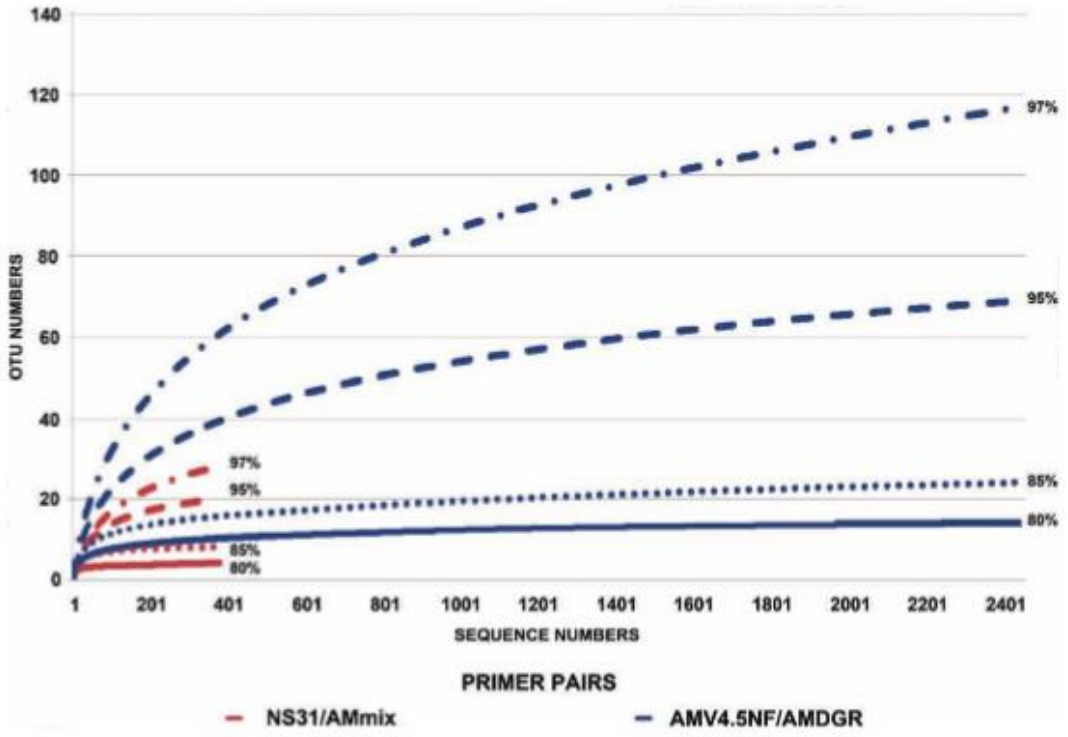

Figure 2. Rarefaction curves for the AMV4.5NF/AMDGR and NS31/AMmix sequences at different similarity levels ranging from 80% to 97%. The 97% similarity level corresponds to $OTU_{0.03}$, the 95% to $OTU_{0.05}$, the 85% to $OTU_{0.15}$ and the 80% to $OTU_{0.20}$.

Table 2. The ability of the two non-parametric richness estimators (ACE and Chao1) to predict the number of *Glomeromycota* OTUs at different levels of similarity in Sardinian soils is compared with the numbers of observed OTUs (rarefaction index).

	Observed OTUs				**Estimated OTUs**							
	Rarefaction				**ACE**				**Chao1**			
Similarity (%)	97	95	85	80	97	95	85	80	97	95	85	80
AMV4.5NF/AMDGR	**117**	**69**	**24**	**14**	**161**	**85**	**33**	**14**	**156**	**79**	**32**	**14**
NS31/AMmix	**28**	**20**	**8**	**4**	**35**	**23**	**9**	**n.c.**	**37**	**25**	**8**	**n.c.**

1. The values are calculated using the DOTUR software for both couples of primers.
2. n.c., not calculated.

The 97% sequence similarity level, corresponding to $OTUs_{0.03}$, was used in the subsequent analyses. The 2438 AMV4.5NF/AMDGR *Glomeromycota* sequences comprised 117 unique $OTUs_{0.03}$, 37 of which were singletons that occurred only once in the entire data set. The remaining 80 OTUs ranged in abundance from 2 to 680 sequences. A total of 377 AMF sequences were obtained with the NS31/AMmix primers and 28 AMF $OTUs_{0.03}$ were generated: nine of these OTUs were singletons, while the other 19 OTUs comprised 2–163 sequences. However, the rank-abundance diagrams (Fig. 3) of both the AMV4.5NF/AMDGR and NS31/AMmix sequences indicated that approximately 50% of the total sequences belonged to three $OTUs_{0.03}$ (OTU numbers 1, 2 and 3 in Table S1) and two $OTUs_{0.03}$ (OTU numbers 1 and 2 in Table S2) respectively.

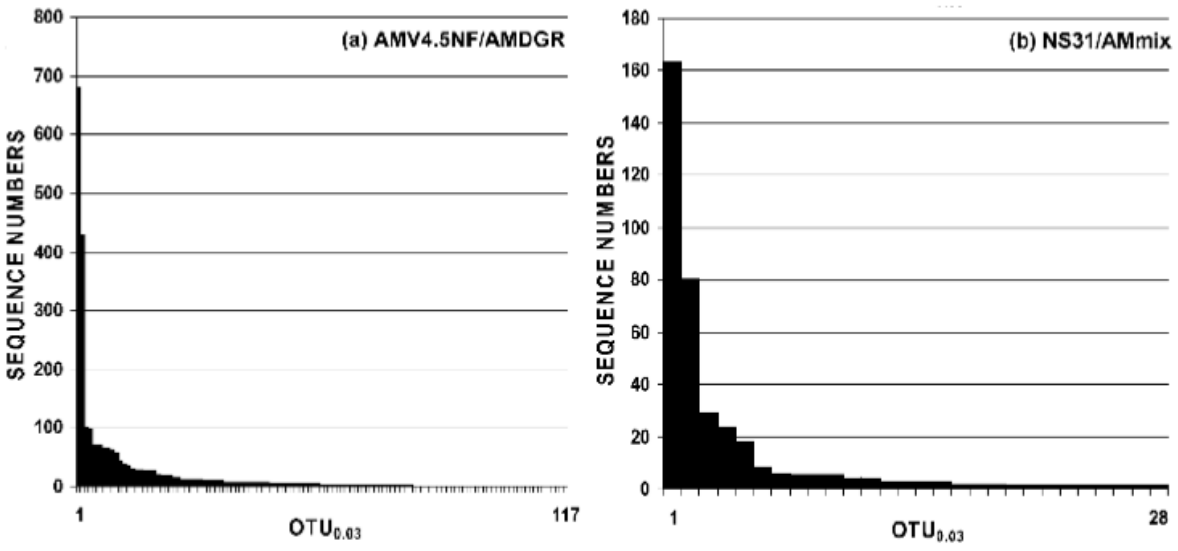

Figure 3. Rank-abundance diagrams (plotting the number of intra-$OTU_{0.03}$ sequences as a function of the OTU rank) for Glomeromycota *$OTUs_{0.03}$ detected with AMV4.5NF/AMDGR (A) and NS31/AMmix (B) at 3% level of dissimilarity. The diagram trend is analogous for both primer sets: on one hand, the first $OTUs_{0.03}$ cover about 50% of all sequences, on the other, a high number of singletons represent the 'long tail' of the diagram.*

The total number of AMF $OTUs_{0.03}$ identified with the two primer pair and their taxonomical distribution over the currently recognized *Glomeromycota* orders are shown in Fig. 4. The ratio of the four AMF orders is almost the same, even though the total number of $OTUs_{0.03}$ generated using the two primer pairs is different: 117 with AMV4.5NF/AMDGR and 28 with NS31-AMmix. As the AMF sequences generated with the NS31/AMmix primers, particularly in the CO and in the TV (12 and 17 sequences respectively), were quite low, the AMF distribution over the five different environments was taken into account considering the sequences generated with the AMV4.5NF/AMDGR primers. Analysing these latter sequences, the number of $OTUs_{0.03}$ generated for each environment was: 27 in the CO (188 total sequences), 30 in the MM (254 sequences), 43 in the CV (292 sequences), 55 in the TV (429 sequences) and 74 in the PA (1275 sequences) (Fig. 5A and B). Figure 6 showed the proportion of the different *Glomeromycota* orders, which was comparable over the Sardinian soils: the *Glomerales* frequencies were always the highest in terms of both the $OTU_{0.03}$ (from 71.6% to 81.5% of the total $OTU_{0.03}$) and sequence numbers (from 81.1% to 95.7% of the total sequences) respectively. In terms of $OTUs_{0.03}$, *Diversisporales* instead represented 18.5% of the CO soil community, 21.6% of the PA, 25.5% of the TV, 25.6% of the CV and 26.7% of the MM of the total sequence numbers (Fig. 6A). In terms of sequences, *Diversisporales* ranged from 4.3% (CO) to 18.9% (MM) (Fig. 6B). *Archaeosporales* and *Paraglomerales* were only found in the PA soil and accounted for 5.4% and 0.5%, and 1.4% and 0.2% of the total $OTUs_{0.03}$ and sequences respectively (Fig. 6A and B).

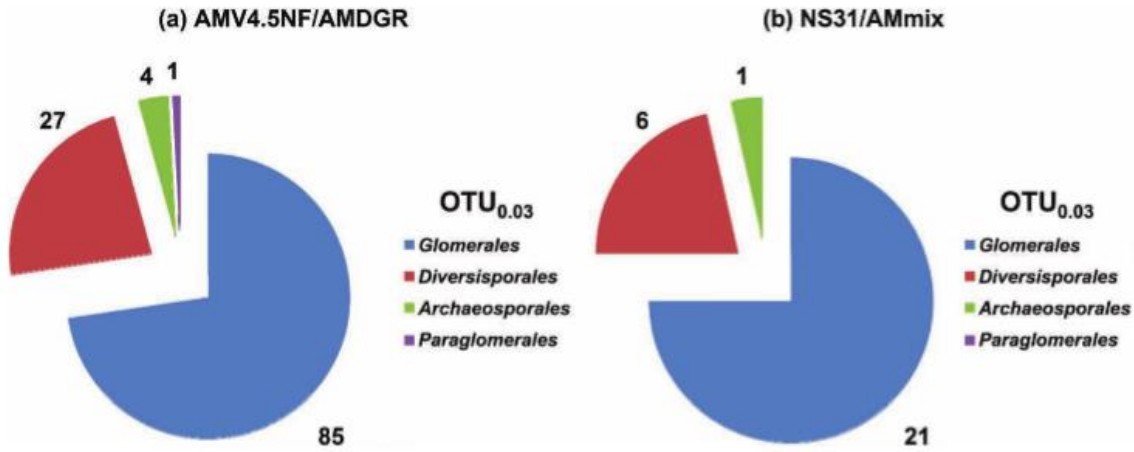

Figure 4. The AMV4.5NF/AMDGR sequences (A) overall generate 117 $OTUs_{0.03}$ and the NS31/AMmix sequences (B) generate 28 $OTUs_{0.03}$. $OTUs_{0.03}$ are separated according to their taxonomy: $OTUs_{0.03}$ belonging to Glomerales *represent the majority for both data sets. An $OTUs_{0.03}$ amenable to* Paraglomerales *(purple) was found with the AMV4.5NF/AMDGR primer set.*

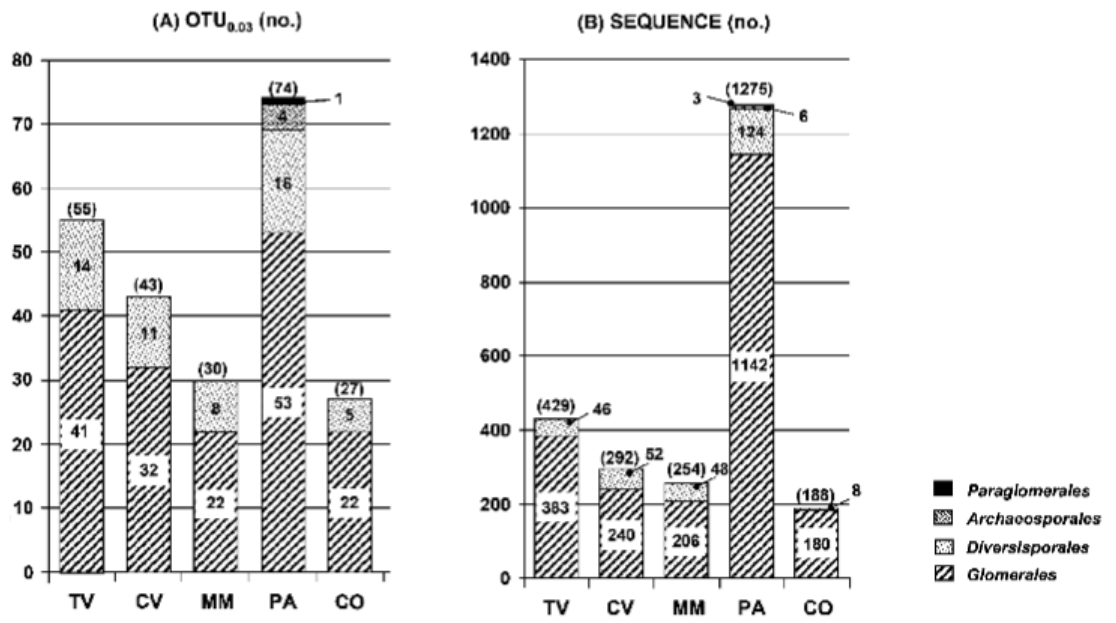

Figure 5. Distribution of different Glomeromycota *orders detected with the AMV4.5NF/AMDGR primer set in each of the five Sardinian environments.*
A. The y*-axis indicates the number of OTUs$_{0.03}$ assigned to each* Glomeromycota *order.*
B. The y*-axis indicates the number of sequences assigned to each* Glomeromycota *order. The total numbers of OTUs$_{0.03}$ and sequences found in each environment are shown in brackets.*
TV, tilled vineyard; CV, covered vineyard; MM, managed meadow; PA, pasture; CO, cork-oak formation.

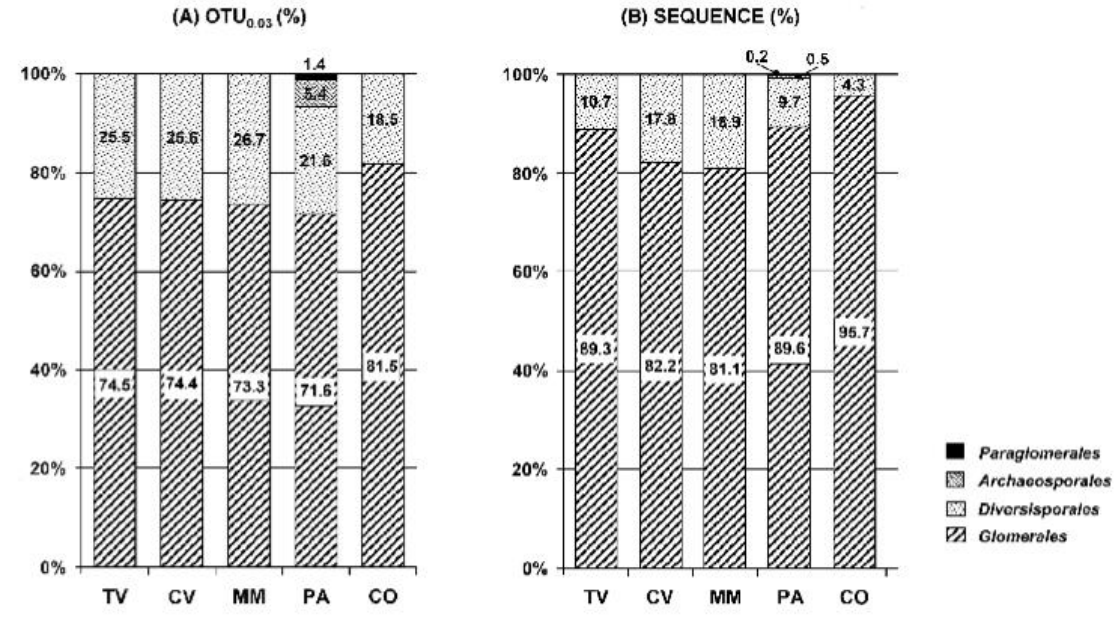

Figure 6. Proportional distribution of the different Glomeromycota *orders detected with the AMV4.5NF/AMDGR primer set in each of the five Sardinian environments.*
A. The y*-axis indicates the proportion of OTUs$_{0.03}$ assigned to each* Glomeromycota *order.*
B. The y *axis indicates the proportion of sequences assigned to each* Glomeromycota *order.*
TV, tilled vineyard; CV, covered vineyard; MM, managed meadow; PA, pasture; CO, cork-oak formation.

A comparison of the observed and estimated $OTUs_{0.03}$ relative to the five soil types, with the non-parametric ACE and Chao1 indices, is shown in Fig. 7. With both indices, the number of observed $OTUs_{0.03}$ is close to the one estimated for the CV. For the other four environments, instead, the number of observed $OTUs_{0.03}$ is always lower than the richness estimated by the ACE and Chao1 indices.

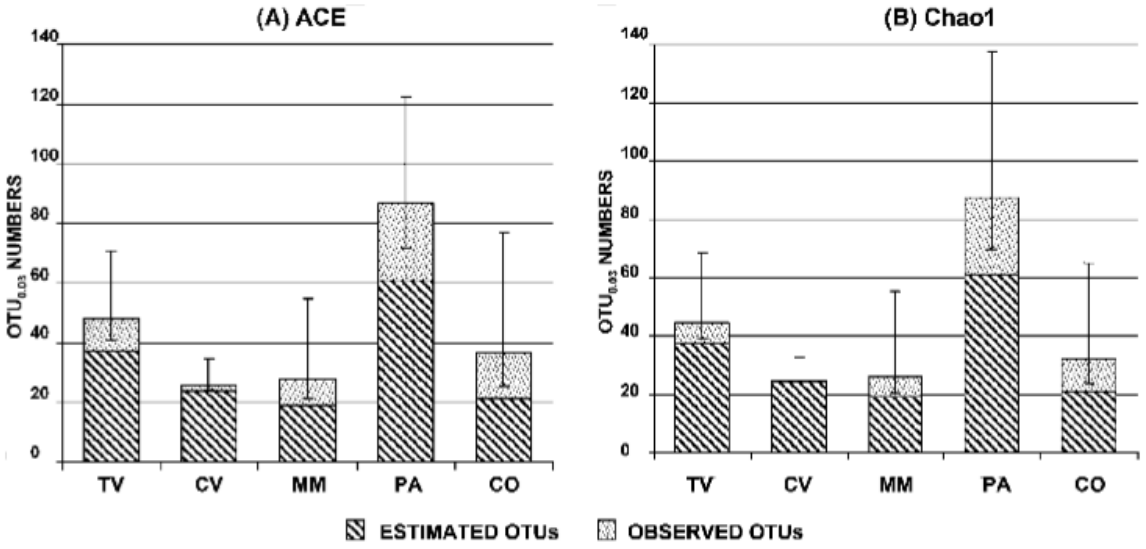

Figure 7. Comparison between the AMV4.5NF/AMDGR $OTUs_{0.03}$ observed and estimated with the two non-parametric estimator indices ACE (A) and Chao1 (B). The bars represent the 95% confidence intervals for the estimated $OTUs_{0.03}$ number. TV, tilled vineyard; CV, covered vineyard; MM, managed meadow; PA, pasture; CO, cork-oak formation.

Shannon–Weaver biodiversity indices (*H′*) were also calculated, taking into account both the number and the relative proportions of taxa in a community, to compare AMF diversity in the different soil types. The *H′* indices for the OTUs detected in each environment with the AMV4.5NF/AMDGR primers at 97% sequence similarity, and the corresponding 95% intervals of confidence are as follows: 1.37 ± 0.23 (CO), 1.55 ± 0.18 (MM), 2.43 ± 0.14 (TV), 2.50 ± 0.13 (CV) and 2.75 ± 0.08 (PA).

Comparison of AMF community memberships and structures

A comparison of the community structures detected using the two couples of primers is shown in Fig. 8. Eleven and one $OTUs_{0.03}$, detected with the AMV4.5NF/AMDGR and NS31/AMmix primers respectively, were shared by all the soils. Among the 11 common $OTUs_{0.03}$ (OTU numbers 1, 2, 4, 13, 15, 16, 19, 23, 24, 28 and 38 in Table S1), nine belonged to *Glomerales* and two to *Diversisporales* and they accounted for 1379 out of 2438 sequences representing 56.6% of the total sequences. The only common $OTUs_{0.03}$ detected by the NS31/AMmix primers (OTU number 1 in Table S2) belonged to *Glomerales* and accounted for 163 out of 337 sequences (44.1% of the total sequences). This $OTUs_{0.03}$ was also the most abundant and commonly identified $OTUs_{0.03}$ when the other primers were used (OTU number 1 in Table S1), on the basis of a pairwise comparison between some sequences of each of these two $OTUs_{0.03}$ (data not shown). These two $OTUs_{0.03}$ (OTU number 1 in Table S1 and in Table S2) most likely correspond to a *Glomus intraradices*-related species.

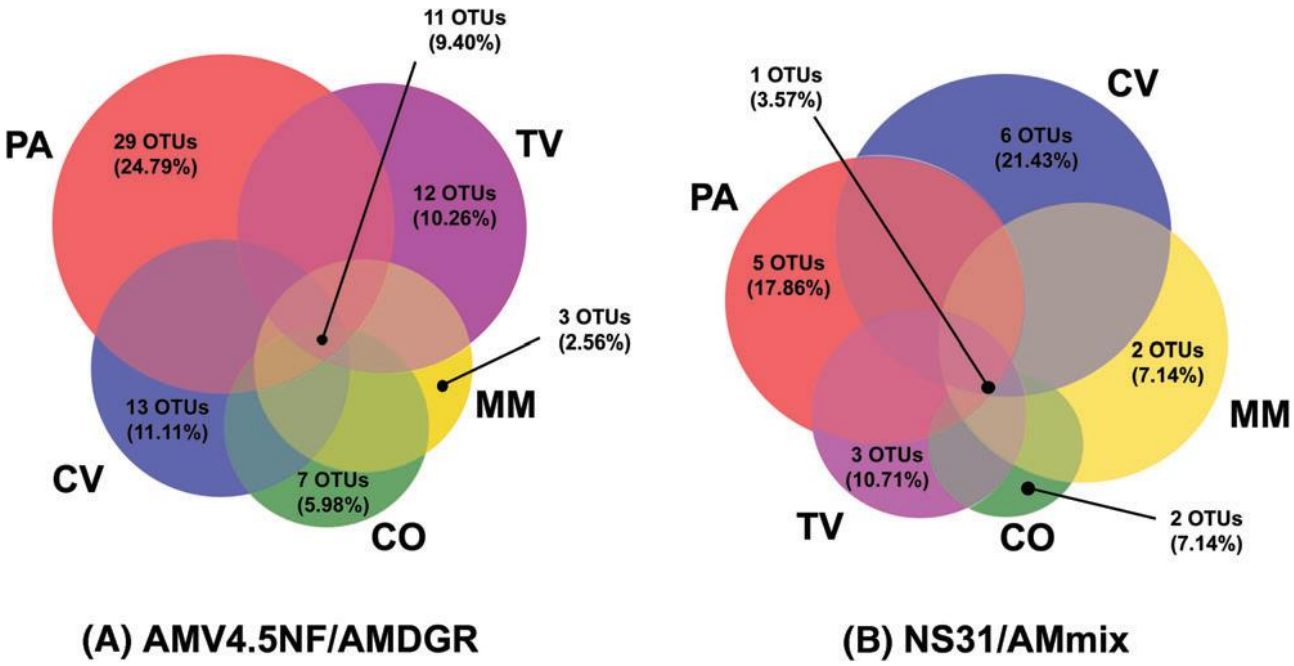

Figure 8. Venn diagrams comparing the $OTUs_{0.03}$ memberships detected with the AMV4.5NF/AMDGR (A) and NS31/AMmix (B) primers in the five land-use soil types. The relative total percentage of $OTUs_{0.03}$ is reported in brackets. The size of each sample component is based on the total number of $OTUs_{0.03}$ for that sample relative to the other samples. TV, tilled vineyard; CV, covered vineyard; MM, managed meadow; PA, pasture; CO, cork-oak formation.

Pairwise Jaccard similarity indices were calculated to evaluate the overlap among the AMF assemblages detected in the five environments with the AMV4.5NF/AMDGR primer pair (Table 3). The TV/PA and TV/CV populations exhibited the highest Jaccard similarity indices: 0.39 and 0.38 respectively. The lowest similarities were found between the cork-oak AMF community and the other four soil populations, with indices ranging from 0.21 (CO/TV) to 0.27 (CO/MM).

Table 3. Jaccard similarity coefficient of AMF communities detected with the AMV4.5NF/AMDGR couple of primers in the five Sardinian soils.

	JACCARD similarity index				
	Tilled vineyard	**Covered vineyard**	**Managed meadow**	**Pasture**	**Cork-oak formation**
Cork-oak formation	0.24240	0.20690	0.26670	0.21690	1
Pasture	0.38710	0.27170	0.30000	1	
Managed meadow	0.34920	0.37740	1		
Covered vineyard	0.38030	1			
Tilled vineyard	1				

1. This index ranges from 0, complete dissimilarity, to 1, complete similarity between two communities. The values reported in the table refer to AMF communities detected at 97% of similarity ($OTUs_{0.03}$).

Discussion

This pyrosequencing study has allowed us to shed light on the diversity of the AMF that thrive in a Mediterranean ecosystem, where different land-use and plant cover types lead to the creation of a soil and vegetation gradient: from an old-growth cork-oak formation to two vineyards (one tilled and one covered with natural plant species), passing through a pasture and a managed meadow. This landscape can be considered of ecological interest as it offers a dynamic mosaic of diverse habitats.

The 454 GS-FLX platform has demonstrated the potentiality of a high-throughput technology to investigate AMF communities and has revealed that 117 *Glomeromycota* OTUs were present in the soils, that *Glomerales* were the dominant order, and – at a lower taxon level – that the *Glomus intraradice*s related species were the overwhelming majority, irrespective of the environment.

Methodological considerations

The first critical point of a metagenomic study, in particular in a complex environment such as soil, concerns the sampling, which should ideally cover the entire biodiversity. In the present work, in order to be sure that representative AMF communities were sampled, we decided to pool the polymerase chain reaction (PCR) products that were independently amplified from the five soil samples from each location (TV, CV, MM, PA and CO). Although this approach does not consider replicates, it offers a reliable overview of the AMF community present in each location (Renker *et al.*, 2006).

Another important aspect that influences the reliability of the results is the technique that is used to analyse the biodiversity. From this point of view, high-throughput technologies, such as pyrosequencing, represents a powerful instrument which is now available in metagenomics. The GS-FLX System potentially produces a huge number of sequences, but due to the design of the experiment and the numerous short reads, only 4192, out of the about 10 000 sequences that were

obtained, were maintained and analysed. Although the number of obtained sequences is lower than that declared for 454 pyrosequencing, it far exceeds the value of fungal sequences obtained until now in fungal biodiversity studies in soil (O'Brien *et al.*, 2005; Hempel *et al.*, 2007). The relatively low number of produced sequences is due to the availability of only five lanes out of 16 in the pyrosequencing plate and to the contemporary sequencing of two amplicon mixtures of different length. In particular, the presence of long (> 300 bp) and short fragments led to a general decrease in the obtainable sequence number, because the long fragments (NS31/AMmix) inhibit the emulsion-PCR step (Margulies *et al.*, 2005) at the expense of short amplicons (AMV4.5NF/AMDGR). The primer pair choice affects the sequence number to a great extent: the AMV4.5NF/AMDGR couple resulted to be the best, in terms of total sequence number, percentage of *Glomeromycota* sequences and spectrum of *Glomeromycota* detected, for AMF soil community analysis. The remarkable difference between sequences obtained with each of the two primer sets is due to the length of the amplified fragments. However, the NS31/AMmix primer set was chosen because a high number of its targeted sequences are deposited in GenBank, even though this primer set did not exactly fit the characteristics required for the GS-FLX pyrosequencing platform. Moreover, a mix of primers AM (AM1, AM2 and AM3) in conjunction with NS31 (Santos-Gonzalez *et al.*, 2007; Toljander *et al.*, 2008) was used in order to target a wider range of AMF taxonomical groups. The obtained results confirmed that this primer set can also amplify non-target organisms (Douhan *et al.*, 2005; Alguacil *et al.*, 2008), especially in a complex environment such as soil, where they have been used for the first time.

A primer pair designed on the SSU region, AML1–AML2 (Lee *et al.*, 2008), which guarantees a good coverage of *Glomeromycota* taxa, is actually available, but it amplifies a fragment of 795 bp, therefore exceeding the suitable length of both the GS-FLX Standard and the New Titanium Series Reagents. Other regions currently used to infer AMF phylogeny were discarded for this metagenomic study because they show some negative aspects. For example, internal transcribed spacer (ITS) and large subunit (LSU) regions, can offer a resolution at a lower taxonomic level (species) than the SSU region. However, it was decided not to use ITS and LSU because of some critical aspects. The ITS region is too variable to be confidentially aligned using 250-bp-long sequences, which is the average length obtainable with the GS-FLX standard kit. For the LSU region the major concern was about the lower number of sequences deposited in GenBank in comparison with the SSU sequences. In conclusion, it seems that a completely satisfactory AMF primer couple is not yet available and that the use of multiple sets is – at the moment – a good strategy to overcome problems.

The limited length (200–300 bp) of fragments that can be obtained with the 454 GS-FLX platform allowed us to identify the AMF at the highest taxonomic levels: we therefore revealed differences among the AMF communities of the five soil types rather than identifying the AMF species. Another difficulty in AMF biodiversity studies at a species level concerns the fact that the majority of AMF sequences deposited in databases are labelled as ‘unknown’ or ‘uncultured’*Glomeromycota*, because they are obtained from molecular analysis of plant roots, without a corresponding morphospecies description. Such a high proportion of ‘known as-sequence-only’ taxa reflects the accumulation of molecular diversity data of AM fungi as a result of the increasing number of studies of *Glomeromycota* in natural ecosystems (Öpik *et al.*, 2008). However, more matches with known AM fungal species could be expected when more effort is directed towards sequencing DNA from morphologically characterized AMF isolates. In the last years, several groups have dedicated a great deal of effort to describing new taxa from spore morphology coupled with molecular analyses (Blaszkowski *et al.*, 2008; Stockinger *et al.*, 2009). In addition, sequencing using new strategies, which lead to longer reads (GS-FLX System with New Titanium Series Reagents), together with new primer availability (Krüger *et al.*, 2009) will allow studies to be conducted at a species level.

As in many other fungal biodiversity studies (Hunt *et al.*, 2004; Santos-Gonzalez *et al.*, 2007), we had to make a critical decision on an appropriate sequences divergence cut-off to define the OTUs. The sequence-based definition of species as clusters of sequences differing by at most 3% of sites was adopted for both the fungi and bacteria. While such a cut-off is generally regarded as canonical for bacterial 16S sequences (Hanage *et al.*, 2006; Konstantinidis and Tiedje, 2007; Elshahed *et al.*, 2008), a similar agreement does not exist for fungal rDNA. In particular, the *Glomeromycota* rRNA gene can show different variants within the same species and even within the same spore (Lanfranco *et al.*, 1999; Sanders, 2004; Börstler *et al.*, 2008). In our study, blast results were found to be consistent for several 97% sequence identity AMF OTUs and we therefore decided to use 97% sequence similarity as the cut-off level. In previous studies, the same sequence identity level was considered a reliable threshold to discriminate possible AMF species (Santos-Gonzalez *et al.*, 2007).

Mediterranean soils harbour diverse AMF assemblages dominated by *Glomerales*

The ecological theory predicts that heterogeneous landscapes should involve higher species diversity levels than homogenous areas since structurally complex habitats provide more niches and ways of exploiting resources (Wardle *et al.*, 2004; Lekberg *et al.*, 2007). Habitat heterogeneity is especially high in typical Mediterranean landscapes (da Silva *et al.*, 2009). Starting from natural ecosystems dominated by cork-oak woodlands, a mix of silviculture and traditional grazing practices has led to a dynamic agro-forest mosaic of different habitats, which might harbour a high below-ground diversity of AMF and consequently shape plant biodiversity and ecosystem functioning (van der Heijden *et al.*, 1998; Maherali and Klironomos, 2007; van der Heijden *et al.*, 2008). Given the increasing interest in soil metagenomes and because, so far, most studies, with a single exception (Hempel *et al.*, 2007), have been based solely on AM spores separated from soil samples and/or mycorrhizal roots, we have explored the potentiality of a 454 GS-FLX platform for a high-throughput study on AMF biodiversity starting from DNA directly extracted from bulk soil, which contains a large proportion of DNA from AM extra-radical mycelium and spores (Johnson *et al.*, 2003; Gryndler *et al.*, 2006). The possibility of studying AMF biodiversity with this new technique is particularly interesting, from an ecological point of view, if we consider that the Sardinian environment is characterized by a land-use gradient, which ranges from old-growth cork-oak woodland to intermediate or high land-use intensification levels, including pasture, vineyards and managed meadows.

The AMF communities of the investigated soils were found to be characterized by a dominance of *Glomerales*. Many studies have reported *Glomus* spp. as the most widespread species in many ecologically different environments: from natural woodlands to high input managed agro-ecosystems (Helgason *et al.*, 2002; Oehl *et al.*, 2005; Hijri *et al.*, 2006). *Glomerales* was the most abundant taxon in terms of $OTUs_{0.03}$ and sequence number: rank-abundance diagrams have indicated that only a few taxa accounted for 50% of the total sequences recovered, while many other $OTUs_{0.03}$ were represented by only a few, or even a single sequence. This suggested a striking dominance of some taxa over many rare taxa (‘tails’ in rank-abundance diagrams). Although some of these rare taxa do possibly really occur, others are likely to be artefacts due to the intrinsic error rate of pyrosequencing, which could lead to an overestimation of the biodiversity, as stated by some recent articles related to 454 pyrosequencing biases. Indeed it has been shown that the presence of long homopolymers in the sequenced fragments may result in frequent miscalls: either insertion or deletions (Quinlan *et al.*, 2008; Kunin *et al.*, 2009). However, interest in developing new algorithms able to manage large data set and eliminating sequencing mistakes is growing in order to overcome this problem (Quince *et al.*, 2009).

The most commonly encountered fungus (OTU number 1 for both primer pairs) possibly corresponds to a *Glomus* species, which is presumably related to the *Glomus intraradices* group (*Glomus* group Ab). *Glomus intraradices* contains several cryptic taxa with differences in various ecological

properties (Croll *et al.*, 2009): this is in agreement with the observation that the AMV4.5NF/AMDGR primer pair amplified sequences that could be amenable to *G. intraradices*, but were grouped in different OTUs. Many isolates of this species have been detected in different locations throughout the world, of both stable and disturbed ecosystems (Öpik *et al.*, 2006; Appoloni *et al.*, 2008) and in many host species (Helgason *et al.*, 2007), suggesting that this AM fungal species has a generalist and ruderal lifestyle (disturbance tolerance) as it produces large numbers of spores and extra-radical mycelium (Jansa *et al.*, 2003; Öpik *et al.*, 2006). Our analysis on soil DNA is in agreement with such behaviour and points out the dominance of this species, not only in plant roots, but also in the soil environment.

The presence, in all five examined soils, of *Diversisporales* $OTUs_{0.03}$, which is thought to produce substantial amounts of external mycelium and dense hyphal clusters (Hart and Reader, 2002) and to prefer sandy soils (Duponnois *et al.*, 2001; Lekberg *et al.*, 2007), is also consistent with both the ecology of this fungal group and the features of the Berchidda area. *Paraglomerales* and *Archaeosporales* were instead only found in the PA soil. Some molecular studies on AM communities (Hijri *et al.*, 2006; Vallino *et al.*, 2006; Alguacil *et al.*, 2008) have reported the difficulty of detecting *Paraglomerales* and *Archaeosporales* in root samples, while Hempel and colleagues (2007) observed that they could be dominant in certain soils. In the current study, only a few $OTUs_{0.03}$ and sequences amenable to these taxa were found and these were only found in the PA soil. This finding would seem to suggest either a scanty occurrence of these orders in the soils or a likely influence of some natural traits, such as those generally associated with grasslands, on the spread of these fungi in ecosystems (Hempel *et al.*, 2007). However, another possible reason for the exclusive detection of *Paraglomerales* and *Archaeosporales* in the PA soil is the high number of sequences obtained from this environment. The high value of the Shannon–Weaver biodiversity index (*H'*), together with the assemblages found in the PA soil, along with the high number of sequences obtained would suggest a higher biodiversity of *Glomeromycota* in the PA, compared with the other four systems. The high AMF diversity in this environment is in agreement with a trend that has already been demonstrated in many studies (Öpik *et al.*, 2006; Öpik *et al.*, 2008): soils subjected to human input (TV and MM) have a lower AMF richness than low-input soils (CV and PA).

As already observed in many bacterial metagenomic studies on soil (Roesch *et al.*, 2007; Fulthorpe *et al.*, 2008), and despite the high number of sequences obtained, in particular with the AMV4.5NF/AMDGR primers, the actual extent of total AMF diversity possibly remains largely undiscovered. This is true for four out of the five environments investigated, while for the CV, the number of observed $OTUs_{0.03}$ is close to the estimated number, indicating good sampling efficiency.

Do AMF communities mirror the vegetation community?

The description of the AMF communities in an area characterized by different land-uses can also allow some considerations to be made on the overlap of the *Glomeromycota* assemblages. Eleven out of the 117 and one out of the 28 $OTUs_{0.03}$ obtained with the two primer pairs were found to be common to all the soils. On the basis of a pairwise comparison between some sequences of each of these two $OTUs_{0.03}$ (data not shown), the only common $OTU_{0.03}$ revealed with the NS31/AMmix probably corresponds to $OTU_{0.03}$ number 1 obtained with AMV4.5NF/AMDGR. These $OTUs_{0.03}$ are likely related to the *G. intraradices* group (*Glomus* group Ab) and they accounted for the highest number of sequences (163 and 680 sequences respectively). This finding confirms the generalistic and likely dominant nature of this group not only in plant roots but also in soil. Despite this overlap, each of the five soil AMF communities exhibited particular features, as indicated by both the occurrence of $OTUs_{0.03}$ unique to each soil type, and the low Jaccard similarity indices obtained in the pairwise comparison.

Of the five AMF communities studied, the CO deserves particular mention. Although the CO is a natural ecosystem, its Shannon–Weaver index was the lowest (H' = 1.37) and the number of AMF $OTUs_{0.03}$ was relatively low, probably because the fungal communities in this environment had shifted from AMF to other mycorrhizal fungal species associated with shrub (*Erica arborea* L and *Arbutus unedo* L) and tree species (*Quercus suber* L). This hypothesis is supported by the high number of sequences belonging to *Sebacinales* (data not shown), a *Basidiomycota* order establishing ericoid mycorrhiza (Selosse *et al.*, 2007), which were obtained with the AMV4.5NF/AMDGR primer pair. The particular composition of the CO soil AMF community was also indicated by the low Jaccard indices obtained when comparing this community with the other four soil AMF communities. This suggests that the plant coverage of this ecosystem, which is characterized by trees and shrubs, has determined not only a reduction in AMF presence but also a typical, unique community structure.

Conclusion

A metagenomic approach applied to AMF fungi and based on a gene of taxonomic interest, like the ribosomal gene, has generated a high number of AMF sequences that far exceeded the number of AMF fungal sequences so far obtained in soil. The obtained data have allowed us to precisely describe the AMF communities that proliferate in a Mediterranean environment, where different land-use and plant cover types lead to the creation of a soil and vegetation gradient. With the exception of a generalist fungus, *Glomus intraradices*, only a few other AMF taxa were found in common in the five environments, suggesting fungal assemblage specificity. The environments with a low input (PA and CV) showed a higher AMF biodiversity than those subjected to human input (MM and TV). A reduction in AMF, replaced by other mycorrhizal fungal species more likely associated to trees and shrubs, was found in the CO. These findings suggest that AMF biodiversity is influenced by both human input and ecological traits. This study poses many questions on the functionality of the AMF communities described, first of all whether the most frequently represented taxa are also the most functionally active. Metagenomic approaches based on soil RNA could help explain not only what species are present, but also what benefits are given to their host plants.

Experimental procedures

Study site

The study area is located in the northern hills of Sardinia, Italy. The Berchidda site (Olbia-Tempio) (40°30′13.37″N 9°47′00.56″E) is made up of hydromorphic and granitic soil with a loamy sand texture. The altitude ranges from 275 m to 300 m. This area is referred to as a mesomediterranean, subhumid phytoclimatic belt with annual rainfall averages of 862 mm (5% summer rainfall percentage), while the mean temperature is 13.8°C (Bacchetta *et al.*, 2004). The soil has pH values that range from 5.0 to 6.5.

In the past, the Berchidda area was covered by cork-oak forests which were subjected to intense usage for the extraction of cork and pasture. Today, there are five dominant soil use types: TV, CV, MM, PA (dominated by grass species with a low tree density) and CO (dominated by shrub cover and distributed cork-oak trees) (Fig. S1).

Soil sampling, DNA extraction, PCR and preparation of the amplicon libraries

In May 2007, five soil core samples (5 cm Ø and 20 cm depth) were taken from each of the five locations. The 25 soil samples were independently packed in ice upon collection and transported to the labs for DNA extraction. The soil samples were sieved (2 mm) to remove fine roots and large

organic debris and stored at −80°C. Twenty-five soil DNA extractions were performed from at least 0.5 g of mixed soil, using the FastDNA Kit (MP Biomedicals, LLC, Fountain Pkwy, Solon, OH, USA).

Two couples of primers: AMV4.5NF/AMDGR (Sato *et al.*, 2005) and NS31 with a mixture of equal amounts of the reverse AM1, AM2 and AM3 (AMmix) (Santos-Gonzalez *et al.*, 2007), were used to amplify an 18S rRNA gene fragment for the 454 GS-FLX pyrosequencing platform. A total of 50 independent PCR amplifications were performed: 25 with AMV4.5NF/AMDGR and 25 with the NS31/AMmix primer set. The DNA amplifications were performed from an equivalent amount of DNA to that found in 2.5 g of each soil type. In order to make sure that the representative AMF communities were sampled, we thought that pooling the PCR products independently amplified from the five soil samples, obtained from the same location (TV, CV, MM, PA and CO), could be the best strategy to overcome the lack of replicates due to the reduced number of available lanes on the pyrosequencing plate (5 out of 16).

The AMV4.5NF/AMDGR primer pair was chosen because it showed suitable characteristics for the GS-FLX System: it amplified a target sequence of approximately 300 bp including the SSU rDNA variable domain V4 from a broad spectrum of *Glomeromycota* (*Glomerales*, *Diversisporales* and *Archaeosporales*) (Sato *et al.*, 2005). Although the NS31/AMmix primers had not been previously used on soil DNA, they were selected because they are frequently used in AMF biodiversity studies: many targeted sequences are therefore easily found in the databases. The general fungal primer AM1 was designed to amplify fungal DNA (Helgason *et al.*, 1998). This primer, in conjunction with the universal eukaryotic primer NS31 (Simon *et al.*, 1992), has been found to amplify AM fungal DNA sequences from field-collected roots (Helgason *et al.*, 1998; Vallino *et al.*, 2006; Alguacil *et al.*, 2008), although it yielded some mismatches at the priming site with taxa belonging to *Glomus* group B and *Glomus* group C (*Diversisporaceae*). For this reason, the exclusive use of AM1 is limiting in a biodiversity study. Therefore, AM2 and AM3 primers were added: these are modifications of the AM1 primer and are designed to amplify DNA from taxa not detected by AM1 (*Glomus* group B and *Glomus* group C) (Santos-Gonzalez *et al.*, 2007; Toljander *et al.*, 2008).

In order to perform 454 pyrosequencing with the GS-FLX System, the sequences of these oligonucleotides included the 454 Life Science A or B sequencing adapters (19 bp) fused to the 5′ primer ends (in brackets):

- A-NS31 5′-(GCCTCCCTCGCGCCATCAG)TTGGAGGGCAAGTCTGGTGCC-3′
- B-AM1 5′-(GCCTTGCCAGCCCGCTCAG)GTTTCCCGTAAGGCGCCGAA-3′
- B-AM2 5′-(GCCTTGCCAGCCCGCTCAG)GTTTCCCGTAAGGTGCCAAA-3′
- B-AM3 5′-(GCCTTGCCAGCCCGCTCAG)GTTTCCCGTAAGGTGCCGAA-3′
- A-AMV4.5NF 5′-(GCCTCCCTCGCGCCATCAG)AAGCTCGTAGTTGAATTTCG-3′
- B-AMDGR 5′(GCCTTGCCAGCCCGCTCAG)CCCAACTATCCCTATTAATCAT-3′

The PCRs contained 17.1 µl of sterile water, 2.5 µl 10× of reaction buffer (Sigma), 2.5 µl of each deoxyribonucleotide triphosphate (dNTP 2.0 µM), 0.5 µl of each primer (10 µM), 0.4 µl of DNA polymerase (High Fidelity Taq, Roche) and 2 µl of DNA template in a final volume of 25 µl.

The DNA was amplified using a T3000 thermal cycler (Biometra, Göttingen, DE). The following programme was used for DNA amplification: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C for 1 min and a final extension at 72°C for 7 min with a ramp of 3°C s^{-1}.

Twenty-five independent amplifications (five for each soil type) were conducted for the AMV4.5NF/AMDGR and NS31/AMmix couples respectively. The PCR products obtained with the two primer pairs were purified with the Agencourt® AMPure® Kit (Beckman Coulter, CA, USA) and pooled to generate 10 samples (five for AMV4.5NF/AMDGR and five for NS31/AMmix). The quality of these samples was assessed through: (i) gel electrophoresis of 5 µl subsamples on 1.5% agarose gel; (ii) evaluation of the AD260/280 ratio calculated using the ND-1000 Spectrophotometer NanoDrop® (Thermo Scientific, Wilmington, DE); and (iii) analysis with the Experion™ System (Bio-Rad, Hercules, CA, USA), using a DNA1K Chip.

In order to create equimolar mixtures of multiple amplicons (amplicon libraries) for 454 pyrosequencing, the 10 pooled samples were quantified by the ND-1000 Spectrophotometer NanoDrop® and five final amplicon libraries (TV, CV, MM, PA and CO), containing 10^{10} molecules/µl of each primer set amplification, were generated. The samples were stored at −20°C and sent to BMR Genomics s.r.l. (Padua, IT) for pyrosequencing by means of a Genome Sequencer FLX System platform (454 Life Science Branford, CT, USA). The samples were processed together with other soils (C. Murat, V. Bianciotto, S. Daghino, M. Girlanda, A. Lazzari, E. Lumini *et al.*, unpublished) and they occupied five lines out of the 16 available in the GS-FLX System.

Alignment, clustering and statistical analyses of the OTU richness

All the sequences were analysed after trimming off the adapter sequences. According to Wommack and colleagues (2008) and after a preliminary blast analysis where small sequences (50–199 bp) did not show any significant similarity, the AMV4.5NF/AMDGR sequences shorter than 230 bp and those shorter than 250 bp were eliminated for the NS31/AMmix primers.

Sequences from the five land use-units were aligned using MUSCLE 3.6 (Edgar, 2004) with default parameters. The alignments were manually edited and distance matrices were constructed using DNAdist from the phylip suite of programmes, version 3.6, with default parameters (Felsenstein, 2005). These pairwise distances were used as input for DOTUR (Schloss and Handelsman, 2005) in order to cluster the sequences into OTUs of a defined sequence identity. The OTUs were defined according to their different sequence similarity values, which spanned 80%–97% sequence identity. Although these distance cut-offs were arbitrary and can be considered controversial, a 97% sequence similarity level, corresponding to 0.03 ($OTU_{0.03}$), was chosen in this study, according to the conventional definition of a microbial 'species' (Rosselló-Mora and Amann, 2001; Konstantinidis and Tiedje, 2007). blast searches were carried out, to test for within-OTU consistency, with sequences within the first 10 most abundant AMV4.5NF/AMDGR and NS31/AMmix OTUs at 97% sequence similarity: these searches yielded the same best blast hit for all the sequences within each OTU. Consensus sequences, obtained for each $OTU_{0.03}$ using CAP3 (Huang and Madan, 1999), were used as queries for the blast searches in the GenBank database. A conservative approach was followed for the fungal species identification, considering only identifications with a ≥ 200 blast score value reliable and labelling all the others as 'unknown organisms'. We used $OTUs_{0.03}$ from DOTUR rather than taxonomic assignments based on blast analyses because not all the sequences matched a known sequence in the database and the use of sequence similarities prevented uncertainties associated with fungal taxonomy and classification.

In order to analyse the richness and diversity of the AM fungal communities in the Sardinian soils, only sequences belonging to *Glomeromycota* were selected for the subsequent analyses and the others were discarded. Arbuscular mycorrhizal fungi sequences were used to generate new distance matrices through DNAdist and these were then used as DOTUR input to generate AMF OTUs containing sequences of different genetic distance values based on a furthest-neighbour algorithm. The use of DOTUR also allowed us to obtain a variety of diversity richness estimators (rarefaction curve, bias

corrected Chao1 richness and abundance-based coverage estimator ACE). Consensus sequences of each AMF $OTUs_{0.03}$ were queried against the GenBank using blast. The blast results were used to calculate the relative abundance of each *Glomeromycota* order within each land-use unit. Similarity among the AMF communities of the five soil-use types was determined using SONS, a software which uses the OTU data obtained from DOTUR to estimate the overlap between pairs of communities and which calculates the Jaccard similarity index (determined as the ratio of the number of OTUs shared and the total number of OTUs in both samples). This index, which ranges from 0 (complete dissimilarity) to 1 (complete similarity), is the simplest parameter to measure the ratio of shared species without considering abundance data.

The Shannon–Weaver diversity index, the rarefaction index and the non-parametric ACE and Chao1 indices were also calculated for each land-use type in order to compare the observed and estimated AMF richness and to assess sampling efficiency in the five soils.

Nucleotide sequence accession numbers

The sequences analysed in this study have been deposited in the EMBL database under accession numbers FN386789–390831.

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